Remarks

Claims 3 and 10 are amended herein. Support for the amendment of claim 3 can be found in the specification at page 7, lines 19-30. Claim 10 is amended to correct dependency.

Claims 8-9 and 11-23 are canceled herein, without prejudice to renewal.

Applicants believe that no new matter is added. Reconsideration of the subject application is respectfully requested.

Information Disclosure Statement

Applicants note that the Examiner has not considered two references (EMBL Accession Number Q12563 and Herscovics *et al.*) that were listed on the Information Disclosure Statement submitted by Applicants on March 25, 2002. For the Examiner's convenience, additional copies of the two references are attached herewith. The Examiner is requested to acknowledge receipt of these additional copies of the references. Applicants believe that no fee is due for the submission of these references, as copies were submitted with the original Information Disclosure Statement.

Specification/Informalities

The trademarks noted by the Examiner have been amended and are capitalized whenever they appear in the specification. Care has been taken to ensure that other trademarks have been similarly identified. Applicants submit that a generic term is associated with each trademark. Applicants submit that the amendments to the specification overcome this objection.

As noted by the Examiner, SEQ ID NO: 18 was incorrect in the previously submitted sequence listing. TAT of SEQ ID NO: 18 encodes tyrosine and not, as previously shown in SEQ ID NO: 18, threonine. Applicants have corrected the obvious error in SEQ ID NO: 18 and submit herewith a corrected version of the Sequence Listing in both paper and electronic versions. Applicants also submit a Statement in Compliance verifying the identity of the paper and electronic versions. Applicants submit that the submission of the corrected sequence listing overcomes the objection.

Rejections under 35 U.S.C. § 101

Claims 3-7, 10-11 and 20-30 under 35 USC § 101, were rejected in the Office action on the basis that the claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility. As noted in the Office action, the claimed polypeptides, encoded by the claimed nucleic acids, are of use for modifying the glycosylation pattern of proteins (see the specification at page 27-28). Applicants submit that Examples 5 and 6 demonstrate a well-established utility for the claimed subject matter. Example 5 outlines two assays that are specific to mannosidase activity, hence demonstrating the proposed activity of the crude culture medium and purified enzyme preparations. A third assay is described in Example 6 in which cell lysates were tested for mannosidase activity.

The Office action seems to contend that because a scientific publication suggests additional experiments, that the present disclosed polypeptides do not have utility. Applicants respectfully disagree.

The utility guidelines are clear: a utility *disclosed in the specification* must be (1) established, (2) asserted by the applicants, (3) specific, and (4) substantial. In the present application, these guidelines have clearly been met. Specifically:

(1) The use is clearly established. For example, the proteins are disclosed to be of use in modifying glycosylation (for example, see the specification at page 27, lines 1-33).

(2) This use is asserted by the Applicants. For example, claim 10 is directed to a method of modifying glycosylation patterns.

(3) The utility is specific. The proteins are disclosed to have a specific function as α-1,2-manosidases (see for example, pages 28-33).

(4) The utility is substantial. There is a real world context of the use of mannosidases, namely to alter properties such as intercellular trafficking, aggregation, and antigenicity of proteins (see the specification at page 3, lines 16-27).

Reconsideration and withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 3-7 and 10 were rejected under 35 USC § 112 as allegedly being indefinite. Claim 3 has been amended to replace "and" with "or," as suggested in the Office action. Claim 10 is

amended to depend from claim 5, as suggested in the Office action. Applicants submit that the amendments of claims 3 and 5 render the rejection moot.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 3-7, 10-11, 20-26 and 28-29 were rejected under 35 USC § 112, first paragraph as allegedly not being enabled. In addition, an assertion was made that there is insufficient written described for these claims. Claims 11-23 are canceled herein. Applicants respectfully disagree with these rejections as applied to the claims as amended.

Claim 3 is amended herein to recited 80% sequence identity. Applicants respectfully disagree with the rejection as applied to the claims as amended. Adequate description is provided by the specification at page 11-15 for the claimed nucleic acids. Moreover, a sequence comparison is provided at page 33-38.

Reconsideration and withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. § 102

Claims 11, 20-23 and 28 were rejected under 35 USC § 102(b) as allegedly being anticipated by GenBank Accession No. AA965900. Claims 11, 20-23 and 28 are canceled herein, rendering the objection moot.

Conclusion

Applicants respectfully request entry of this amendment. If any matters remain to be addressed, please contact the undersigned at the telephone number listed below.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By

Susan Alpert Siegel, Ph.D.
Registration No. 43,121

One World Trade Center, Suite 1600
121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 226-7391 / Facsimile: (503) 228-9446

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 13, Issue of April 1, pp. 9864-9871, 1994
Printed in U.S.A.

Isolation of a Mouse Golgi Mannosidase cDNA, a Member of a Gene Family Conserved from Yeast to Mammals*

(Received for publication, September 8, 1993, and in revised form, November 29, 1993)

Annette Herscovics‡§, Jean Schneikert‡, Ariadni Athanassiadis‡, and Kelley W. Moremen¶‖

From the ‡McGill Cancer Centre, McGill University, Montréal, Québec H3G 1Y6, Canada and the ¶Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

The amino acid sequence of the specific α-mannosidase involved in *N*-oligosaccharide processing in *Saccharomyces cerevisiae* was found to have a high degree of similarity to the deduced amino acid sequence of a rabbit liver α-mannosidase partial cDNA, demonstrating that processing mannosidases have been conserved through eukaryotic evolution. Regions of sequence identity were chosen to design degenerate oligonucleotide primers that can be used to prepare probes using the polymerase chain reaction (PCR) for cloning processing mannosidases from other eukaryotes. Using these primers for PCR with mouse liver cDNA as template, two related but distinct PCR products were obtained. The amino acid sequences of PCR_1 and PCR_2 were 88 and 65% identical with the corresponding sequence of the rabbit enzyme, respectively. Southern blot analysis of mouse genomic DNA using PCR_1 and PCR_2 as probes revealed that they are derived from two different genes, indicating the existence of a mammalian mannosidase gene family with at least two members. Using PCR_2 as a probe, a novel mouse cDNA was isolated from a 3T3 cDNA library. It contains an open reading frame which encodes a type II membrane protein of 73 kDa with a cytoplasmic region of about 35 amino acids, a Ca^{2+} binding consensus sequence, and a single *N*-glycosylation site. Northern blot analysis of mouse tissues and L cells revealed tissue-specific expression of multiple transcripts, ranging in size from 4.2 to 8.5 kilobases, that suggests a complex pattern of gene regulation. Transient expression of the influenza hemagglutinin epitope-tagged cDNA in COS cells followed by indirect immunofluorescence with monoclonal antibody 12CA5 showed that the cloned mannosidase is primarily localized in a juxtanuclear position corresponding to the Golgi. The C-terminal domain lacking the putative transmembrane region was shown to have α-mannosidase activity when expressed in COS cells as a secreted Protein A fusion product.

Mannosidases play an important role at different stages in the maturation of *N*-oligosaccharides in mammalian cells. This pathway begins with the transfer of a dolichol-linked oligosaccharide precursor, usually $Glc_3Man_9GlcNAc_2$, to Asn/*X*/Ser(Thr) on newly formed polypeptide chains (for review, see Kornfeld and Kornfeld (1985)). $Glc_3Man_9GlcNAc_2$ is then trimmed by two specific glucosidases which remove the glucose residues, and by several ER[1] and Golgi α1,2-mannosidases that can cleave up to 4 mannose residues to yield $Man_5GlcNAc_2$. The action of α1,2-mannosidases at this point in the pathway is an essential step in the maturation process, for the resulting $Man_5GlcNAc_2$ can be modified by GlcNAc transferase I, the first glycosyltransferase leading to the synthesis of hybrid or complex oligosaccharides. Subsequently, Golgi α-mannosidase II can remove the terminal α1,3- and α1,6-linked mannose residues from $GlcNAcMan_5GlcNAc_2$ to form $GlcNAcMan_3GlcNAc_2$. This oligosaccharide is then further modified by Golgi glycosyltransferases to generate the variety of *N*-complex structures found on glycoproteins.

Studies with inhibitors clearly demonstrate the essential role played by mannosidases in the maturation of *N*-oligosaccharides (for review, see Elbein (1991)) and show that preventing the activity of processing mannosidases may have important biological consequences. The α1,2-mannosidase inhibitors, 1-deoxymannojirimycin and kifunensine, which inhibit the synthesis of *N*-complex oligosaccharides and cause the accumulation of oligomannose oligosaccharides, have been shown to interfere with the development of capillaries *in vitro* (Nguyen *et al.*, 1992). The α-mannosidase II inhibitor, swainsonine, which causes the formation of hybrid structures instead of complex oligosaccharides, is able to reverse the transformed phenotype of NIH 3T3 cells *in vitro* (DeSantis *et al.*, 1987), and to inhibit tumor cell metastasis *in vivo* (Dennis, 1986; Newton *et al.*, 1989). Although little is known of the molecular genetics of human processing mannosidases, one form of the human hereditary anemia, HEMPAS is caused by a deficiency in α-mannosidase II expression (Fukuda, 1990). As a result, hybrid oligosaccharides are found on HEMPAS erythrocyte glycoproteins in place of the normal polylactosamine structures, an alteration that causes increased susceptibility to lysis.

Biochemical studies indicate that several processing α1,2-mannosidases exist in mammalian cells with different molecular properties, specificities, and subcellular localization, but the number of distinct mannosidases and their respective role in the processing pathway is not known (for review, see Moremen *et al.* (1994)). Cloning of mammalian α1,2-mannosidases is therefore necessary to determine how many of these enzymes are involved, to establish their specific role in the maturation process and their intracellular localization, and to elucidate the genetic control of the early stages of *N*-oligosaccharide processing.

* This work was supported by a research grant from The Medical Research Council of Canada and by National Institutes of Health Grant GM31265. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is dedicated to the memory of Gersz Nejman (1906–1942).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U03457 and U03458.

§ To whom correspondence should be addressed: McGill Cancer Centre, 3655 Drummond St., Montréal, Québec H3G 1Y6, Canada. Tel.: 514-398-3533; Fax: 514-398-6769.

‖ Present address: Dept. of Biochemistry and the Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602.

[1] The abbreviations used are: ER, endoplasmic reticulum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; bp, base pair(s); kb, kilobase pair(s); ORF, open reading frame; MOPS, 4-morpholinepropanesulfonic acid.

TABLE I
Oligonucleotide primers used for PCR reactions

Oligonucleotides were designed to amino acid sequences conserved between the yeast and rabbit mannosidases. Oligonucleotide 1 corresponds to the yeast amino acid residues 275–283, and oligonucleotide 2 to amino acid residues 528–523 (Camirand *et al.*, 1991). Amino acids are above the nucleotide sequences in one-letter code, with the sequence reversed for the antisense oligonucleotide. Nucleotide sequences are written 5′ → 3′. For degenerate codons either a mixture of nucleotides were used as indicated, or nucleotides containing inosine (I). The residues in *italics* represent additional nucleotides to generate restriction sites for subcloning (1, *Eco*RI and 2, *Hin*dIII).

Oligonucleotide 1 (sense)	*CGC*	*GAA*	*TTC*	D GAT C	S TCI AG	F TTT C	Y TAT C	E GAA G	Y TAT C	L TTI C	L TTA C G C T	K AA
Oligonucleotide 2 (antisense)		*CGC*	*AAG*	*CTT*	H ATG G	A AGC G C T	E TTC C	T TGT G A C	N ATT G	F AAA G		

In contrast to mammalian cells, the trimming process is much simpler in the yeast, *Saccharomyces cerevisiae*. The early stages of *N*-oligosaccharide biosynthesis, including glucose removal, are identical to those observed in mammalian cells, but there is only one processing α1,2-mannosidase that removes a single mannose residue from the middle arm of the precursor oligosaccharide to form a single isomer of $Man_8GlcNAc_2$. The oligosaccharide precursor is then elongated by Golgi mannosyltransferases to form the mature mannoproteins characteristic of *S. cerevisiae* (for review, see Herscovics and Orlean (1993)). The yeast specific α-mannosidase has been purified (Jelinek-Kelly *et al.*, 1985; Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991) and its gene (*MNS1*) has been isolated (Camirand *et al.*, 1991). It encodes a type II membrane protein of 63 kDa containing a very short cytoplasmic region of 2–3 amino acids, three *N*-glycosylation sites, a calcium-binding consensus sequence (Camirand *et al.*, 1991), and a catalytic domain facing the lumen of the endoplasmic reticulum (Grondin and Herscovics, 1992). In contrast to mammalian cells where preventing mannosidase activity interferes with the formation of complex oligosaccharides, disruption of the yeast processing mannosidase gene is of little consequence to the subsequent maturation of *N*-oligosaccharides in *S. cerevisiae* (Puccia *et al.*, 1993).

Although in recent years, an increasing number of mammalian glycosyltransferase genes and cDNAs have been cloned and characterized (for reviews, see Schachter (1991), Shaper and Shaper (1992), and Joziasse (1992)), little is known of the molecular genetics of processing glycosidases. No processing glucosidase and only two full-length mannosidase cDNAs have been isolated from mammalian cells. The first is a rat liver cDNA encoding a cytosolic/ER α-mannosidase that can remove mannose residues from $Man_{9-5}GlcNAc$ (Shoup and Touster, 1976; Bischoff and Kornfeld, 1983, 1986). The role of this cytosolic/ER enzyme in the processing pathway needs to be clarified since no hydrophobic region which could serve as transmembrane domain or signal sequence is found in the deduced amino acid sequence (Bischoff *et al.*, 1990). This absence is unusual for an enzyme expected to act on oligosaccharide processing on the lumenal side of the ER. In fact, this cDNA is homologous to the yeast vacuolar α-mannosidase, a membrane-bound enzyme acting in the lumen of the yeast vacuole, that gains access to this compartment by a signal sequence-independent mechanism (Yoshihisa and Anraku, 1990). The other processing mannosidase cDNA clone that has been reported encodes Golgi α-mannosidase II (Moremen and Robbins, 1991). The cDNA contains an unusually long 3′-untranslated region and encodes a protein whose deduced amino acid sequence exhibits the type II membrane topology characteristic of Golgi glycosyltransferases.

In the present work we show that the derived amino acid sequence of the yeast processing mannosidase (Camirand *et al.*, 1991) exhibits significant similarity (37% identity, 58% similarity) to the rabbit liver Ca^{2+}-dependent α1,2-mannosidase partial cDNA briefly described by Moremen *et al.* (1990). This cDNA was isolated using amino acid sequence obtained from the rabbit liver α1,2-mannosidase purified by Forsee *et al.* (1989). The yeast and rabbit α1,2-mannosidases have no apparent similarity with the amino acid sequences of either Golgi α-mannosidase II (Moremen and Robbins, 1991) or the cytosolic/ER α-mannosidase (Bischoff *et al.*, 1990). Regions of identical amino acid sequences between the yeast and the rabbit enzyme were chosen to design degenerate oligonucleotides for PCR on mouse liver cDNA as template. Using the resulting PCR products, we present evidence for the existence of two related mouse mannosidase genes, and report the isolation of a novel mouse mannosidase cDNA that exhibits tissue-specific expression. We also show, using epitope tagging, that this mannosidase is localized to a juxtanuclear position corresponding to the Golgi following transient expression in COS cells.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: restriction enzymes, New England Biolabs, Life Technologies Inc., or Pharmacia LKB Biotechnology Inc. (Baie D'Urfé, Québec); the GenAmpDNA amplification reagent kit, Perkin Elmer Cetus; Sequenase, U. S. Biochemical Corp.; T7 polymerase sequencing kit, Sepharose 6B, IgG Sepharose 6FF, Pharmacia, LKB Biotechnology Inc.; the Cyclone Biosystem M13 deletion kit, International Biotechnologies Inc.; the random primed DNA labeling kit, U. S. Biochemical Corp.; Zetaprobe membranes, Bio-Rad; Hybond-N nylon membrane, Amersham Corp. All other reagents were at least reagent grade. Synthetic oligonucleotides were prepared at the MIT Biopolymers Laboratory on an Applied Biosystems (Model 380B) DNA synthesizer, or at the Sheldon Biotechnology Centre, McGill University, on a Gene-Assembler Plus from Pharmacia according to the manufacturer's instructions. Plasmid preparations were obtained using columns obtained from Qiagen Inc. Bovine serum albumin (highest grade) was obtained from Boehringer-Mannheim (Laval, Quebec). All procedures were performed according to Sambrook *et al.* (1989) unless otherwise specified.

Polymerase Chain Reaction Experiments—Degenerate oligonucleotides corresponding to two regions that were completely conserved between the yeast (Camirand *et al.*, 1991) and the rabbit (Moremen *et al.*, 1990) mannosidases were designed as shown in Table I. The sense oligonucleotide contained 2 deoxyinosine residues. First strand cDNA was synthesized from oligo(dT)-selected mouse liver RNA using murine leukemia virus reverse transcriptase and random primers, as described previously (Moremen, 1989). This cDNA served as a template in the polymerase chain reaction using the Perkin Elmer Cetus reagents in a final volume of 50 μl containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each dNTP, 1 μM of each oligonucleotide primer, and 2.5 units of Taq polymerase, overlaid with 50 μl of mineral oil. 35 automated step cycles were conducted as follows: 1 min at 92 °C, 1 min at 50 °C, and 3 min at 72 °C. The last cycle was

followed by a 3-min extension at 72 °C. Control PCR reactions were also done with rabbit liver cDNA and yeast genomic DNA as templates. The products were fractionated by electrophoresis in 1.6% agarose. A major amplification product of about 760 bp was obtained with all three templates. The PCR product obtained from mouse liver cDNA was subcloned into *Eco*RI/*Hin*dIII-digested M13 mp18 and M13 mp19 for sequencing of 10 random clones in each orientation. Two populations of sequences, named PCR_1 and PCR_2, were obtained.

Isolation of cDNA Clones—A BALB/c 3T3 cDNA library primed with a mixture of oligo(dT) and random hexamers and packaged into a λZAP II vector (Stratagene, La Jolla, CA) was obtained from D. J. G. Rees (Massachusetts Institute of Technology) (Rees *et al.*, 1990). The 672-bp PCR_2 product was random-labeled (specific activity, 2–4 × 10^9 cpm/μg of DNA) and used to screen 2.6 × 10^5 plaque forming units of the cDNA library spread on a lawn of XL1-Blue cells (35 × 150-mm Petri dishes). Plaque lifts were carried out in duplicate using Hybond-N nylon membranes, followed by prehybridization and hybridization with labeled PCR_2 (6–8 × 10^5 cpm/ml) as probe, as previously described (Moremen and Robbins, 1991). Positive clones were purified by three additional rounds of screening as described above. Excision of the pBluescript plasmid containing the cloned cDNA from λZAP II was done by coinfection with M13 K07 helper phage, according to the manufacturer's instructions, except that the XL1-Blue cells were grown in LB.

DNA Sequencing—Sequencing was done mostly in M13 by the "dideoxy" chain termination method (Sanger *et al.*, 1977) using Sequenase version 2.0, as described by the manufacturer. Regions of compression were resequenced using 7-deaza-dGTP or dITP. Successive deletions were done in M13 using T4 polymerase (Cyclone Biosystem M13 deletion kit, IBI, New Haven, CT). In some cases, confirmation on the opposite strand was obtained using synthetic oligonucleotides for sequencing of Bluescript clones. Sequence assembly was done using the SeqMan program of DNASTAR (Madison, WI).

Southern Blot Analysis—Mouse genomic DNA was digested overnight at 37 °C with restriction enzymes and fractionated by electrophoresis in a 1.2% agarose gel. The gels were treated successively with 0.25 M HCl for 15 min, with 0.5 M NaOH, 1.5 M NaCl for 30 min, and with 0.5 M Tris-HCl, pH 8, 1.5 M NaCl for 30 min, rinsing with water between solutions. The gel was equilibrated in 10 × SSC and transferred to Hybond-N in 10 × SSC for 1 h using a pressure blotter (Posiblot, Stratagene). Following transfer, the DNA was cross-linked to the membranes by exposure to UV light (Stratalinker, Stratagene). Prehybridization was performed for 1 h at 65 °C in a solution containing 5 × SSC, 5 × Denhardt's, 0.2% SDS, and heat-denatured salmon sperm DNA. Hybridization was done overnight at 65 °C in a solution containing 5 × SSC, 10 × Denhardt's, 0.4% SDS, 10 mM EDTA and either random-labeled PCR_1 or PCR_2 (2 × 10^6 cpm/ml, specific activity, 2–4 × 10^9 cpm/μg of DNA). The filters were washed twice for 15 min at room temperature, and twice for 15 min at 65 °C in 2 × SSC, 0.2% SDS.

Northern Blot Analysis—Total RNA from different adult BALB/C mouse tissues and from L cells (the TK^- $APRT^-$ line also known as LTA cells) was isolated using guanidinium thiocyanate extraction followed by centrifugation through a CsCl cushion essentially as described by Chirgwin *et al.* (1979). Poly(A^+) mRNA was isolated using the PolyATtract mRNA MagneSphere system III from Promega according to the manufacturer's instructions.

The poly(A^+) RNA was denatured at 65 °C in 30% formamide, 10% formaldehyde in 10 mM MOPS buffer, pH 7, and fractionated by electrophoresis in 1% agarose/formaldehyde gels overnight at 40 V, followed by transfer to Hybond-N for 2 h on a vacuum blotter (VacuGene, Pharmacia). The RNA was cross-linked to the membrane using UV light (Stratalinker). Prehybridization was done for 1 h at 65 °C in 0.5 M Na_2PO_4 buffer, pH 7, containing 1 mM EDTA, 7% SDS, 10 mg/ml bovine serum albumin, 100 μg/ml denatured herring sperm DNA. Hybridization was performed overnight at 65 °C in the same solution without herring sperm DNA, and containing random-labeled cDNA probe derived from the ORF of clone 4 (3 × 10^6 cpm/ml, specific activity 1 × 10^9 cpm/ml). The blots were washed as previously described (Moremen and Robbins, 1991). An RNA ladder (Life Technologies Inc., Burlington, Ontario) was used as standards. The probe for glyceraldehyde-3-phosphate dehydrogenase, a "housekeeping" gene, was used to monitor the quantity of RNA.

Subcloning in Expression Vectors—For intracellular expression in COS cells using pXM-139 (Yang *et al.*, 1986) the entire coding region was isolated using PCR with the sense 5′-oligonucleotide *CAT CTCGAG CCACC* ATG ACT ACC CCA GCG containing an *Xho*I site and a Kozak consensus sequence upstream from the initiation codon, and the 3′ antisense oligonucleotide *CCG CTCGAG* TCA TCG GAC AGC AGG ATT ACC containing an *Xho*I site downstream from the stop codon. Additional constructs were prepared appending the sequences corresponding to the influenza HA epitope, YPYDVPDYAS, onto the 3′ end of the ORF for epitope tagging (Field *et al.*, 1988; Kolodziej and Young, 1989). Clone 4 and a reconstituted clone 4/16 in which the *Bam*HI-*Not*I 3′ fragment of clone 16 was ligated to the 5′ *Not*I-*Bam*HI of clone 4 were used as template (100 ng) for PCR (20 cycles with Taq polymerase: 1 min at 94 °C, 1 min at 44 °C, 5 min at 72 °C). The constructs were sequenced.

For expression of the mannosidase cDNA as a secreted Protein A fusion protein, the pPROTA plasmid containing the IgG binding domain of *Staphylococcus aureus* Protein A fused to the transin signal peptide (Sanchez-Lopez *et al.*, 1988) was used essentially as described previously for expression of glycosyltransferases (Larsen *et al.*, 1989; Kukowska-Latallo *et al.*, 1990). The vector was first modified by insertion of a *Kpn*I adaptor in its unique *Eco*RI site to yield plasmid pPak. The C-terminal region of clone 4/16 was isolated using PCR with the sense 5′ oligonucleotide *C GTG GTA CCG* CGT CTG AGA AAT AAG ATT AG containing a *Kpn*I site before nucleotide 904 of the cDNA (corresponding to amino acid 106) and the 3′ antisense oligonucleotide *GCA GGT ACC* TCA TCG GAC AGC AGG ATT ACC containing a *Kpn*I site downstream from the stop codon. PCR was performed with Taq polymerase (25 cycles of 1 min at 94 °C, 1 min at 37 °C, 4 min at 72 °C). The resulting PCR product was cut with *Kpn*I and subcloned into pPak to produce plasmid pPakman 416/106.

Transfection of COS Cells for Immunofluorescence—COS 7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% glutamine, 1% penicillin/streptomycin. The day before transfection, the cells were trypsinized and seeded onto four-chamber glass slides (Nunc, Naperville, Il). Cells at 50–70% confluency were transfected by the DEAE-dextran plus chloroquine method (Ausubel *et al.*, 1989) with 1 μg/chamber of mannosidase cDNA in pXM-139. Expression was allowed to proceed for 20–64 h before the cells were processed for immunofluorescence, essentially as described (Lejbkowicz *et al.*, 1992). Cells were washed twice in PBS, fixed for 30 min at room temperature in 4% formaldehyde in PBS, permeabilized for 30 min at room temperature in 0.2% Tween 20, 4% formaldehyde, PBS. After washing with 0.2% Tween 20, PBS, nonspecific sites were blocked by incubation for 60 min at room temperature in fetal calf serum supplemented with 10% goat serum, 6% skim milk, 3% bovine serum albumin, 0.2% Tween 20, 0.02% NaN_3. After washing with 0.2% Tween 20, PBS, the cells were incubated overnight at 4 °C with a 1:1000 dilution of mouse ascites fluid containing 12CA5 monoclonal antibody directed against the influenza HA epitope. The cells were washed five times with 0.2% Tween 20, PBS, and then incubated for 1 h at room temperature in the dark with a 1:100 dilution of tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse IgG (H+L) (Jackson Immunoresearch, West Grove, PA). Following seven washes in 0.2% Tween 20, PBS, slides were mounted in 30% glycerol, 0.02% NaN_3, PBS, sealed with nail polish, and kept in the dark. Cells were viewed with a Zeiss IM35 inverted microsocope with epifluorescence. Photographs were taken on Kodak Tmax 400 film with an MC 100 camera at either 100 or 400 × magnification.

Transfection of COS Cells for Secretion of Fusion Protein—Plasmids pPakman416/106 and pPak were separately transfected into COS 7 cells maintained in 100-mm dishes containing 10 ml of the supplemented Dulbecco's modified Eagle's medium, as described above using the DEAE-dextran plus chloroquine method with 6 μg of expression vector per plate. The medium was harvested 64 h after transfection, a mixture of protease inhibitors (2 μg/ml each of pepstatin A, leupeptin, and chymostatin) was added followed by centrifugation at 2000 × *g* for 10 min. The medium from each dish was concentrated 100-fold using Centri-cell 20 ultrafilters with 30,000 M_r cut-off (Polysciences Inc., Warrington, PA). The resulting concentrate was diluted 10-fold with PBS and first preadsorbed by mixing with 250 μl of Sepharose 6B (50% slurry in PBS) for 2 h at 4 °C. The Sepharose 6B beads were discarded following centrifugation and the supernatant was then adsorbed by mixing with 200 μl of IgG Sepharose 6FF (50% slurry in PBS) overnight at 4 °C. The beads were collected and washed three times with 10 volumes of buffer (50 mM Tris, pH 7.6, containing 150 mM NaCl and 0.05% Tween 20) and three times with 50 mM potassium phosphate buffer, pH 6.0, containing 1 mM $CaCl_2$. For assay of α-mannosidase activity, 40 μl of the beads (50% slurry in the same phosphate buffer) was incubated with 7.5 μl of uniformly labeled [^{3}H]$Man_9GlcNAc$ (8200 cpm) prepared as described previously (Jelinek-Kelly *et al.*, 1985) and 56 μg of bovine serum albumin for 4.5 h at 37 °C. The amount of [^{3}H]mannose released was measured in the supernatant following precipitation with concanavalin A, as described previously (Herscovics and Jelinek-Kelly, 1987).

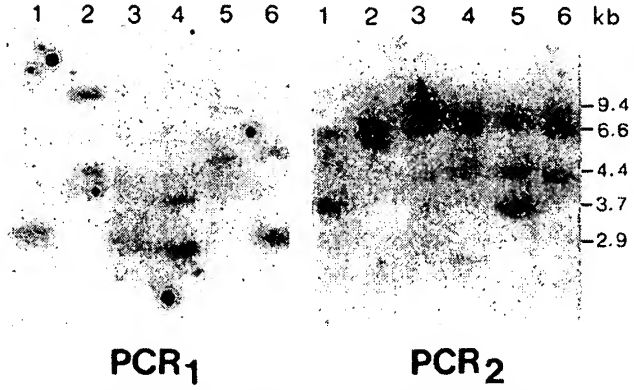

FIG. 1. **Southern blot of mouse genomic DNA.** Duplicate samples of mouse genomic DNA digested with: *1*, *Eco*RI; *2*, *Pst*I; *3*, *Bam*HI; *4*, *Xba*I; *5*, *Hin*dIII; *6*, *Bgl*II were probed with random labeled PCR_1 or PCR_2, as indicated.

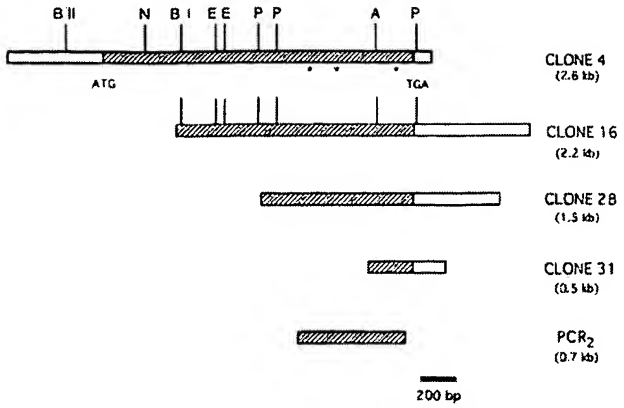

FIG. 2. **Restriction map of isolated cDNA clones.** The relationship between the four clones isolated from the 3T3 cDNA library and PCR_2 is shown. *Shaded areas* correspond to regions in the ORF. The *asterisks* indicate single base differences between clone 4 (C) and the other clones (T). These code for Thr, Leu, Ser in clone 4, compared to Met, Phe, Phe in the other clones, from 5′ to 3′. Restriction sites are: *BII*, *Bst*EII; *N*, *Nco*I; *BI*, *Bam*HI; *E*, *Eco*RI; *P*, *Pst*I; *A*, *Acc*I.

RESULTS

Sequence Similarity between Yeast and Rabbit Mannosidases—The deduced amino acid sequence of the *MNS1* gene (accession number, M63598) encoding the yeast processing α-mannosidase that removes a single specific mannose residue from $Man_9GlcNAc$ (Camirand *et al.*, 1991) was found to be similar to that of a partial cDNA encoding an α1,2-mannosidase purified from rabbit liver (Forsee *et al.*, 1989; Moremen *et al.*, 1990, accession number V04301). The deduced amino acid sequences of these two enzymes exhibited 37% identity and 58% similarity when analyzed with the Bestfit (version 7) sequence analysis program from the University of Wisconsin Genetics Computer Group. Four regions of 7–10 amino acids (amino acids 139–145, 274–283, 501–509, and 523–528 of the yeast mannosidase, see Camirand *et al.* (1991)) are identical in the yeast and rabbit mannosidases. Two of these conserved peptide sequences were used to design degenerate oligonucleotides as primers (Table I) for PCR on templates of rabbit liver cDNA, mouse liver cDNA, and the rabbit mannosidase cDNA clone as control. In all cases a product of about 760 bp was obtained, corresponding to the size expected for amplification between the two primers. Restriction analysis, however, suggested that the PCR product obtained using mouse liver cDNA as template might be heterogeneous. The mouse liver PCR product was therefore subcloned into M13 in both orientations and random clones were sequenced. Two PCR populations with different sequences were obtained: the deduced amino acid sequence of PCR_1 was 88% identical with the corresponding region in the rabbit mannosidase cDNA clone and 98% identical with that of a mannosidase cDNA clone isolated from a 3T3 cDNA library using the rabbit mannosidase cDNA as a probe.[2] On the other hand, the amino acid sequence of PCR_2 exhibited only 65 and 72% amino acid sequence identity with the rabbit and its corresponding 3T3 mannosidase cDNA, respectively. Both PCR_1 and PCR_2 contained a third conserved region corresponding to amino acids 501–509 of the yeast mannosidase sequence.

Southern Blot Analysis—Since the isolation of two distinct PCR products suggested that there may be two different mouse mannosidase genes, Southern blots of mouse genomic DNA hybridized with labeled PCR_1 and PCR_2 were compared (Fig. 1). It is evident that the pattern of labeled restriction fragments obtained with these two probes is quite different, thereby demonstrating the existence of two distinct mannosidase genes.

Isolation of cDNA Clones Encoding Murine Mannosidase—Labeled PCR_2 was used as a probe to screen a 3T3 cDNA library. Four independent clones were obtained from approximately 2.6×10^5 recombinants: clone 4 (2.6 kb), clone 16 (2.2 kb), clone 28 (1.5 kb), and clone 31 (0.5 kb). These clones were excised from the λZAP II vector, and subcloned into M13 for sequencing. Clones 4 and 16 were sequenced completely on both strands, and were shown to overlap, as indicated in Fig. 2. The overlapping regions have identical nucleotide sequences except for positions 1232, 1402, and 1775 of the ORF which are C in clone 4 and T in clone 16, causing changes in amino acids from Thr to Met, Leu to Phe, Ser to Phe, in these positions, respectively. The combined sequences of clones 4 and 16 consist of 3.2 kb (Fig. 3) and contain an ORF of 1926 bp flanked by a long 5′-untranslated region of 589 bp, and a 3′-untranslated region of about 700 bp. Since no poly(A) tract or consensus polyadenylation signal is found, the combined clones 4 and 16 are still missing some 3′-untranslated region to correspond to the shortest transcript of 4.2 kb observed on Northern blots (see later in Fig. 6).

The putative α-mannosidase ORF corresponding to the PCR_2 probe encodes a protein of 641 amino acids (M_r 72,938). The methionine codon in position 1 is in a favorable context for initiation of translation since it is surrounded by an A at positions −3 and +4 (Kozak, 1989). There is an upstream termination codon in frame with the putative initiation codon and separated from it by three bases. The 5′-untranslated region is long (589 bp) and G/C rich, predicting considerable secondary structure. A major hydrophobic region between amino acids 37 and 58, close to the N terminus, is a putative transmembrane domain, suggesting that this mannosidase is a type II membrane protein, with a cytoplasmic domain of about 35 amino acids. The immediate N-terminal sequence next to the hydrophobic region has a net positive charge compared to the immediate C-terminal region, in accordance with the predicted topology (von Heijne and Gavel, Y., 1988; Hartman *et al.* 1989). There is a single potential *N*-glycosylation site close to the C terminus, and a putative 12 amino acid Ca^{2+} binding consensus sequence at positions 255–266 (Marsden *et al.*, 1990).

The deduced amino acid sequence of the C-terminal region (from amino acid 161) of the mouse α-mannosidase cDNA corresponding to PCR_2 exhibits 37% identity and 60% similarity with the yeast processing α-mannosidase (from amino acid 22) (Fig. 4). In this region of similarity 3 cysteine residues (amino acids 462, 494, and 565 of the mouse sequence) are conserved in both proteins, as well as the 12-residue calcium binding con-

[2] A. Lal, J. S. Schutzbach, W. T. Forsee, P. Neame, and K. W. Moremen, manuscript submitted for publication.

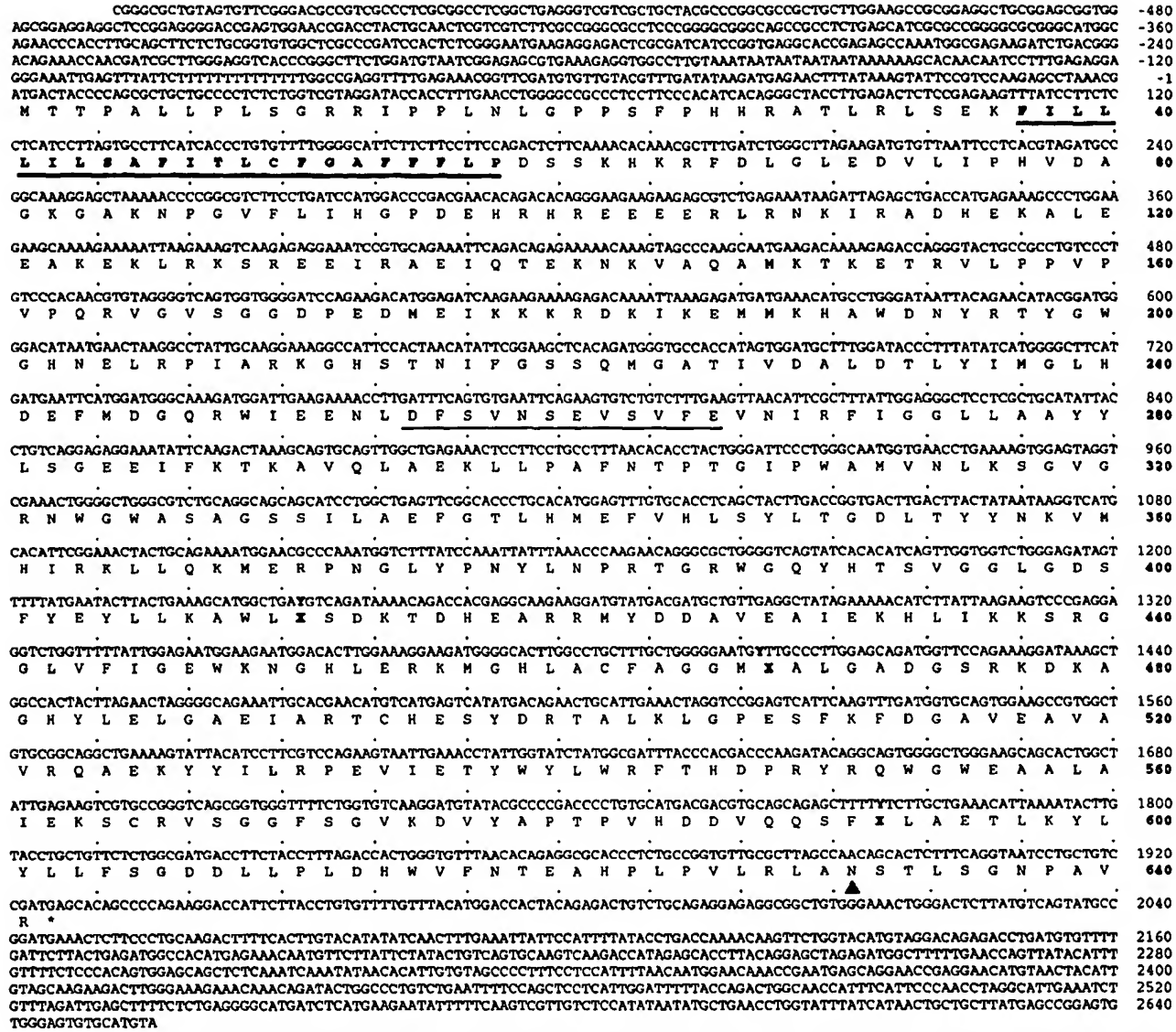

FIG. 3. **Nucleotide and deduced amino acid sequence of mouse mannosidase cDNA.** The nucleotide sequence of the combined clone 4 and clone 16 cDNAs is shown. On the right the *numbers* in *normal type* refer to the nucleotides with position 1 corresponding to the initiation codon while those in *bold* refer to numbering of the amino acids indicated by the single letter code under the nucleotide sequence. Single base differences between clone 4 and clone 16 are indicated by *Y* at positions 1232, 1402, and 1775 which are C in clone 4 and T in clone 16 giving the following amino acid differences indicated by *X* at positions 411, Thr or Met, 468, Leu or Phe, 592, Ser to Phe, for clones 4 and 16, respectively. The *underlined* sequence in *bold* is the hydrophobic region corresponding to the putative transmembrane domain. The *underlined* sequence in *plain type* is the 12-amino acid consensus Ca^{2+}-binding sequence. ▲, represents the single potential *N*-glycosylation site.

sensus sequence. The sequons for *N*-glycosylation, however, are not conserved. The N-terminal regions of the yeast and mouse mannosidases are very different. The mouse protein has a cytoplasmic region of about 35 amino acids and about 100 amino acids between its putative transmembrane domain and the region of homology that are not present in the yeast mannosidase. On the other hand, the deduced amino acid sequences of the two mouse mannosidases corresponding to PCR_1 and PCR_2 exhibit 64% identity and 77% similarity with each other throughout their whole sequence, as determined by the Bestfit program. The dot plot comparing the two mouse amino acid sequences is shown in Fig. 5.

Expression of Mannosidase in Mouse Tissues—Northern blots of mouse tissues show a complex pattern of expression following hybridization with the coding region of clone 4 under stringent conditions. Several transcripts ranging in size from about 4.2 to 8.7 kb were observed, with tissue-specific patterns in their relative expression (Fig. 6). The highest level of expression was observed in L cells, followed by colon, ovary, thymus, and brain; lower levels were observed in kidney, uterus, liver, and lung. In most cases the major transcripts were 4.2, 5.1, 6.4, and 8.7 kb, with the notable exception of brain in which the major transcript was 8.7 kb, and ovary which also had a high level of expression of the 5.6 kb transcript. A Northern blot of L cells showed a similar pattern of transcripts using the 3′-untranslated region, 5′-untranslated region, PCR_2, or the entire coding region as probes (data not shown).

Expression of Mannosidase in COS Cells—Transient expression of the epitope-tagged mannosidase cDNA in COS cells followed by indirect immunofluorescence using monoclonal antibody 12CA5 to the influenza hemagglutinin epitope showed strong immunofluorescence in a juxtanuclear position in a majority of positive cells (Fig. 7) 24–64 h after transfection. In some cells there was also a fine reticular pattern of immuno-

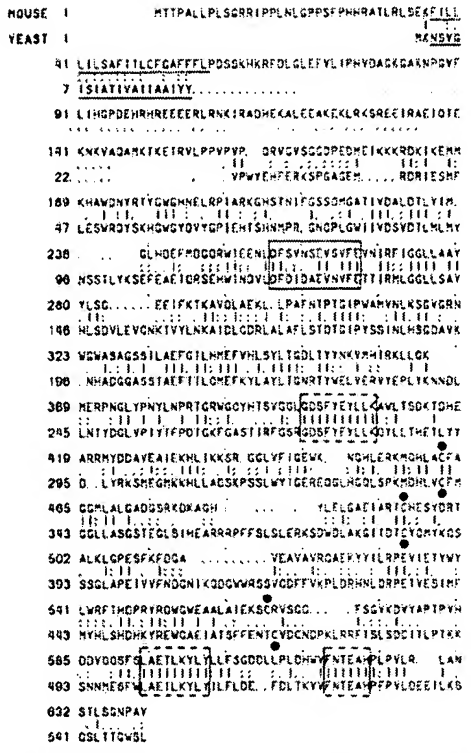

FIG. 4. **Alignment of mouse mannosidase corresponding to probe PCR_2 and yeast processing mannosidase protein sequences.** The Bestfit (Version 7) sequence analysis program from the University of Wisconsin Genetics Computer Group was used for the alignment (gap weight = 3.0, gap length weight = 0.1), except that the putative transmembrane domains of the two proteins (*underlined*) were lined up. The mouse mannosidase amino acid sequence is on top and the yeast processing mannosidase is on the bottom. *Bars* indicate amino acid identities, *double dots* indicate conservative amino acid substitutions, and *single dots* indicate similar amino acid substitutions. ●, conserved cysteine residues; *rectangles* surround regions conserved between species, the *solid* one being the Ca^{2+}-binding consensus sequence.

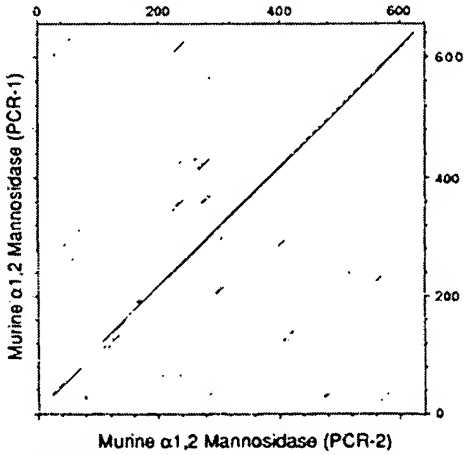

FIG. 5. **DOTPLOT analysis.** The mouse α-mannosidase corresponding to probe PCR_2 was compared to the mouse α-mannosidase corresponding to probe PCR_1 (Lal *et al.*, Footnote 2; accession number V04299) Comparisons were made with the DOTPLOT program from the University of Wisconsin Genetics Computer Group, Version 7, using a window of 30 and a stringency of 15.

fluorescence corresponding to the ER, indicating an accumulation of the mannosidase in the ER most likely due to overexpression. There was no apparent difference in the immunofluorescence pattern observed with clone 4 or a reconstituted clone 4/16.

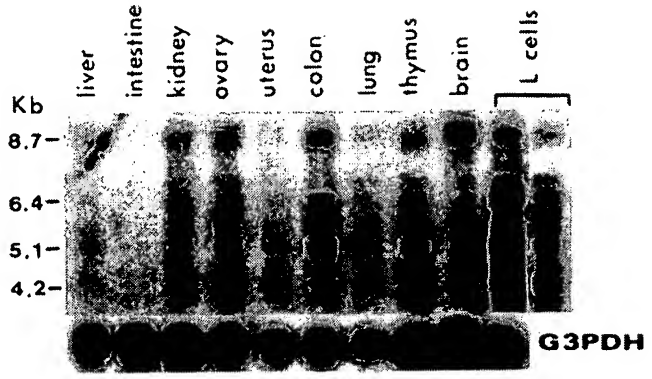

FIG. 6. **Northern blot of mouse tissues and L cells.** Poly(A$^+$) RNA (10 μg) from mouse tissues and L cells were probed with random labeled coding region of clone 4. L cells showed a similar pattern of transcripts using random labeled PCR_2, 5'-untranslated region (nucleotides −558 to −226), or 3'-untranslated region (nucleotides 1995 to 2656) as probe (see Fig. 3). Exposure for radioautography to Kodak X-AR5 film was 2 days, except for the RNA from L cells in the last lane which was re-exposed overnight. *G3PDH*, the blot was probed with glyceraldehyde-3-phosphate dehydrogenase cDNA.

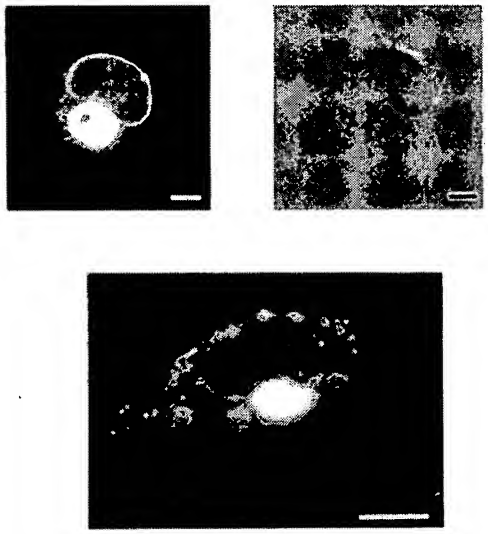

FIG. 7. **Localization of mannosidase.** Epitope-tagged mouse mannosidase cDNA was localized in COS 7 cells by immunofluorescence using monoclonal antibody 12CA5 to the influenza hemagglutinin epitope, as described under "Experimental Procedures." The *top panel* shows immunofluorescence (*left*) and phase-contrast of the same cell (*right*) 64 h post-transfection and the *bottom panel* shows immunofluorescence of a cell 42 h post-transfection. The *bars* indicate 3 μm.

To establish that the isolated cDNA encodes a catalytically active mannosidase, the C-terminal region of clone 4/16 lacking the putative transmembrane domain was expressed in COS cells as a secreted fusion protein with the IgG binding domain of *S. aureus* Protein A in the mammalian expression vector pPROTA (Sanchez-Lopez *et al.*, 1988) essentially as described previously for the expression of glycosyltransferases (Larsen *et al.*, 1989; Kukowska-Latallo *et al.*, 1990). The medium of cells transfected with either the control vector pPak, or the vector encoding the fusion protein pPakman 416/106 was treated with IgG-Sepharose, and the α-mannosidase activity of the beads was assayed directly using uniformly labeled [^{3}H]$Man_5GlcNAc$ (8200 cpm) as substrate, as described previously (Herscovics and Jelinck-Kelly, 1987). In separate transfections, no significant release of [^{3}H]Man was observed in the samples obtained from cells transfected with the control vector compared to about

420, 485, and 570 cpm of [^{3}H]Man released from three different samples obtained from cells transfected with the vector encoding the fusion protein. These results indicate that the soluble form of clone 4/16 encodes an enzymatically active α-mannosidase. Additional work will be necessary to establish the specificity of this enzyme.

DISCUSSION

This report is the first demonstration of the existence of a mannosidase gene family conserved through eukaryotic evolution. The similarity observed between the amino acid sequence of the processing α-mannosidase from *S. cerevisiae* and the rabbit liver Ca^{2+}-dependent α-mannosidase allowed us to design degenerate oligonucleotide primers for PCR which are useful to isolate members of this gene family from different species. Using these degenerate primers for PCR with mouse liver cDNA as template, two distinct but related PCR products (PCR_1 and PCR_2) were obtained. Southern blot analysis of mouse genomic DNA using PCR_1 and PCR_2 as probes revealed that the mouse genome contains at least two members of the mannosidase gene family.

A novel mouse mannosidase cDNA was isolated using PCR_2 as a probe. It encodes a type II membrane protein of 73 kDa which localizes to the Golgi following transient expression in COS cells. Its derived amino acid sequence is very similar to that of the mouse mannosidase cDNA corresponding to PCR_1[2] that was isolated using the rabbit liver cDNA clone as a probe (Moremen *et al.*, 1990). The two mouse enzymes are highly similar in sequence (64% identity, 77% similarity), size, and topology. They both have a Ca^{2+} binding consensus sequence. The N-terminal region of the mouse mannosidases is completely different from that of the yeast mannosidase. The yeast protein has a different transmembrane region and lacks a significant cytoplasmic region as well as the region of about 100 amino acids immediately following the transmembrane domain. It will be of interest to determine whether the differences in the N-terminal region of the mouse and yeast enzymes are related to different subcellular localization or to differences in enzyme specificity. It has been demonstrated that the transmembrane domain is essential for targeting of glycosyltransferases to the Golgi (for review, see Shaper and Shaper (1992)). The yeast processing mannosidase, unlike the two mouse mannosidases, is thought to be located in the ER or in an intermediate pre-Golgi compartment, since the *sec18* mutant, which is blocked in ER to Golgi transport, is capable of trimming *N*-oligosaccharides to $Man_8GlcNAc_2$ at the nonpermissive temperature (Esmon *et al.*, 1984).

The complex pattern and tissue-specific expression of the mouse mannosidase observed upon Northern blot analysis may be due to a combination of different factors including alternate splicing, the use of different polyadenylation sites, and of alternate tissue-specific promoters. In some tissues, there is an inverse relationship between the expression of the two mannosidase genes. For example, liver which expresses the highest level of mRNA hybridizing with PCR_1, has a low level of expression with PCR_2, whereas L cells which express high levels of PCR_2 have no detectable PCR_1 transcripts. Recent studies on the regulation of expression of the β1,4-galactosyltransferase gene which specifies two different transcripts indicate that these arise from differential initiation from alternate promoters in a tissue-specific manner (Harduin-Lepers *et al.*, 1993). Studies on the organization of the mannosidase gene will be required to understand its transcriptional regulation.

It is difficult to determine whether the cDNA described in the present work encodes any of the processing α1,2-mannosidases that have been characterized previously. The first of these enzymes to be characterized was Golgi α-mannosidase I (Tabas and Kornfeld, 1979; Tulsiani *et al.*, 1982). It was purified from rat liver and further resolved into two components termed Golgi α-mannosidase IA and IB based on differences in their elution upon ion-exchange chromatography (Tulsiani *et al.*, 1982; Tulsiani and Touster, 1988). The two enzymes have similar, but not completely identical substrate specificities. α-Mannosidase IA was purified to homogeneity and shown to have a subunit molecular mass of 57 kDa, but it is not known whether this subunit represents the complete gene product since membrane-bound glycosidases and glycosyltransferases are usually purified as proteolytically released soluble proteins lacking their N-terminal region. Antibodies to α-mannosidase IA were shown to cross-react with α-mannosidase IB, and EM immunolocalization studies with these antibodies showed that α-mannosidase IA was present in medial Golgi in NRK and Chinese hamster ovary cells, medial and *trans*-Golgi in rat pancreatic acinar cells and enterocytes, and across the entire Golgi stack in rat hepatocytes (Velasco *et al.*, 1993). These results show that α-mannosidase IA distribution varies from one cell type to another and is less compartmentalized than previously assumed. No effect of exogenous Ca^{2+} on rat liver Golgi mannosidase I was reported, but the effect of Ca^{2+} was not tested in the presence of EDTA. The possibility that Ca^{2+} is required for enzyme activity cannot be ruled out since the requirement of the yeast mannosidase for Ca^{2+} could only be demonstrated following inhibition with EDTA (Jelinek-Kelly and Herscovics, 1988). From these studies, it is therefore not possible to determine whether the cDNA isolated in the present work encodes the previously described Golgi α-mannosidase IA/IB.

Calcium-dependent α1,2-mannosidases have been purified from rabbit (Forsee and Schutzbach, 1981; Forsee *et al.*, 1989), calf (Schweden *et al.*, 1986), and pig (Schweden and Bause, 1989; Bause *et al.*, 1992) liver. Although differences in specificity of these mannosidases were reported, they all remove α1,2-linked mannose residues from oligosaccharide and glycoprotein substrates. The most recent study indicates that the differences were due to the use of different substrates and that the rabbit and pig liver enzymes are immunologically related (Bause *et al.*, 1992). The pig liver enzyme, however, was localized to the endoplasmic reticulum and not to the Golgi of pig hepatocytes by immunoelectron microscopy (Roth *et al.*, 1990). If the cDNAs corresponding to PCR_1 and PCR_2 encode enzymes related to the pig and calf liver enzymes, there must be considerable cell type or species-dependent variation in subcellular localization of mannosidases since transient expression of the cDNA corresponding to PCR_2 shows a Golgi localization.

Calcium-independent α-mannosidases α1,2/1,3/1,6-mannosidases have been isolated from different tissues (Shoup and Touster, 1976; Bischoff and Kornfeld, 1983, 1986; Tulsiani and Touster, 1985; Bonay and Hughes, 1991), but these are clearly different from the protein encoded by the cDNA reported in the present work since their subunit molecular size is much larger (107–110 kDa). In some cells, an endomannosidase capable of cleaving glucose containing oligosaccharides is also present in the Golgi (Lubas and Spiro, 1987), but this enzyme has not yet been purified. It is evident therefore that additional work will be necessary to determine the role of the cloned mannosidases in the processing pathway and to establish their relationship with the previously described Golgi and Ca^{2+}-dependent mannosidases.

Acknowledgments—One of us (A. H.) thanks Dr. P. W. Robbins for hospitality and valuable suggestions while on sabbatical leave in his laboratory, partly supported by a "Bourse de perfectionnement en biotechnologie" from the Fonds de la Recherche en Santé du Québec and by National Institutes of Health Grants GM31318 (to P. W. R.) and GM 47533 (to K. W. M.). We thank Barry Sleno and Izabella Lejbkowicz for

excellent technical assistance, Dr. Nicole Beauchemin for stimulating discussions, and Drs. Brent Weston and John Lowe for providing the vector pPROTA.

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